Tropical Ecology **58**(1): 193–198, 2017
© International Society for Tropical Ecology
www.tropecol.com

ISSN 0564-3295

No copper required for germination of an endangered endemic species from the Katangan Copperbelt (Katanga, DR Congo): *Diplolophium marthozianum*

SYLVAIN BOISSON*, WILLIAM ORTMANS, JUSTINE MARÉCHAL, MARIE MAJERUS, GRÉGORY MAHY & ARNAUD MONTY

Biodiversity and Landscape Unit, Biosystems Engineering Department (BIOSE) – Gembloux Agro-Bio Tech, University of Liege, B–5030 Gembloux, Belgium

Abstract: Two hypotheses were tested with respect to the germination of *Diplolophium marthozianum,* an endemic plant species of the copper-cobalt outcrops in Katanga, Democratic Republic of Congo: (1) germination of copper-endemics is limited by fungal infection in the absence of pathogen control and (2) the germination success of this copper-endemic species is copper-dependent. Seed lots of twenty seeds were weighed, soaked in different disinfection treatments and then placed in a germination medium containing four distinct copper concentrations for 30 days. Seed viability was measured at the beginning and at the end of the experiment by a cut test. Final germination percentage (15.2 ± 8.2%) and seed viability (24.2 ± 10.3%) were not affected by copper concentration or disinfection treatments. *D. marthozianum* is able to germinate in a substrate without added copper, despite pervasive fungal infection. However, seed mass had a significant positive effect on seed germination suggesting that selecting the largest seeds may ensure the highest germination success in *ex situ* conservation programs.

Key words: Conservation, disinfection, metallophyte, pathogen, seed, threatened taxa, trace metal.

Handling Editor: Juan Manuel Dupuy

The Global Strategy for Plant Conservation aims to halt the continuing loss of plant diversity. Understanding, documenting and developing conservation strategies for plant species are key issues that need to be urgently addressed as international responsibilities (UNEP 2007). Biodiversity conservation priorities should focus especially on tropical regions, which include 90% of extant species (Myers *et al.* 2000). Rare endemic taxa are considered priorities for conservation programs when threats are identified (Primack 2010; Schemske *et al.* 1994).

Metalliferous areas (i.e. soils with elevated concentrations of trace metals) often host rare ecologically endemic taxa due to the severe selection pressures induced by trace metals, founder effects and genetic drift induced by the insularity of these habitats (Baker *et al.* 2010).

Corresponding Author; e-mail: sylvain.boisson@ulg.ac.be; sylvainboisson@hotmail.com

Plants that are distributed only on metal-rich soils are called absolute metallophytes (Pollard *et al.* 2002). Many of these species are threatened by mining, which leads to degradation or even total destruction of their habitats, increasing extinction risks (Saad *et al.* 2012; Whiting *et al.* 2002).

The Katangan Copperbelt (Southeastern Democratic Republic of Congo) is formed by more than 150 copper-cobalt outcrops, which are globally unique (Duvigneaud & Denaeyer-De Smet 1963). These hills are geographically and ecologically isolated and have a unique flora that includes over 600 species, including 32 taxa that are strict endemic species (i.e. occurring solely on Cu-rich soils) or absolute cuprophytes (e.g. *Crepidorhopalon perennis*), and 24 taxa that are broad endemic species (i.e. more than 75% occurrence on Cu-rich soils) (Faucon *et al.* 2010). Many copper-endemic species are highly threatened by habitat destruction caused by industrial mining (Faucon *et al.* 2010).

Many factors influence the success of current *ex situ* conservation efforts focused on reproduction (Di Salvatore *et al.* 2008; Godefroid *et al.* 2013). Chipeng *et al.* (2010) showed that copper concentrations or the disinfection of seeds prior to germination trials may improve germination percentage for the absolute cuprophyte *Haumaniastrum katangense* (S. Moore) P. A. Duvign. & Plancke. The regeneration of metallophytes poses particular problems because of their limitation to highly metal-enriched substrates. This limitation may be due to various factors. First, these species may have higher copper physiological needs than other species (Faucon *et al.* 2012; Peng *et al.* 2012). Second, copper may limit the development of pathogens, e.g. by acting as a natural fungicide (Antonovics *et al.* 1971); thus, metal hyperaccumulation can provide an effective form of defence (called elemental defence) against a wide range of pathogens (Boyd 2007; Fones *et al.* 2010). From an evolutionary point of view, the metallophytes may have therefore lost their pathogen-resistance mechanisms (Tadros 1957). Third, these species may also be limited to metalliferous soils because of inter-specific competition; e.g. endemics could be less competitive in other environments but able to grow on non-metal-enriched soils in the absence of competition (i.e. edaphic endemism, Gankin & Major 1964).

In order to develop a rapid conservation strategy and maintain maximum genetic diversity, the scientific community seeks to reproduce copper-endemics from seed collections rather than by cloning methods. However, there is still no standardized protocol for managing seed lots of these species from the wild, and it is unclear which disinfection and other specific treatments have to be applied to seeds in order to enhance their germination.

The aim of this study is to provide guidelines for *ex situ* conservation of endemic species from copper-rich ecosystems. The first critical process of a plant propagation strategy is seed germination; therefore, this study focuses on germination. Two hypotheses regarding seed germination of an endemic species from copper ecosystems, *D. marthozianum*, were tested. First, germination of this copper-endemic species is limited by fungal attack in the absence of pathogen control by copper. Second, the germination success of this species is copper-dependent. In addition, the effect of seed mass on germination was also examined to determine if seeds should be sorted by size prior to germination in conservation programs.

Diplolophium marthozianum P. A. Duvign. (Apiaceae) is a perennial cuprophyte and a broad endemic species with a high affinity for Cu–Co rich substrates in Katanga (Faucon *et al.* 2010; Saad *et al.* 2012). Its proposed classification for the *IUCN Red List of Threatened Species* is endangered (EN; Faucon *et al.* 2010). Like many plants of the Apiaceae family, seed teguments have microcavities on their surface, where fungal pathogens can shelter (Bulajic *et al.* 2009). The achenes of *D. marthozianum* (henceforth referred to as seeds) used in the present experiments were collected on three sites in the Katangan Copperbelt between Tenke (10.61°S; 26.12°E) and Fungurume (10.62°S; 26.32°E) towns; one collection was made in July 2011 at Mwinansefu, another in July 2011 at Fungurume IV and the third seed lot was collected in September 2011 at Kavifwafwaulu. Seeds were stored in paper bags at room temperature, in Gembloux, Belgium, for 5 to 15 weeks before germination tests.

Germination tests were performed in Petri dishes under sterile conditions on a culture medium consisting of agar (0.8%) (Kumari & Ichhpujani 2000). Copper in the form of copper (II) sulphate ($CuSO_4$) was used. Previous studies on seed germination of non-metallophyte species have shown that the phytotoxicity of copper appears between 2.5 and 10 mg l^{-1} Cu, whereas the absolute cuprophyte *H. katangense* (which occurs in the Katangan Copperbelt) tolerates more than 10 mg l^{-1} Cu (Chipeng *et al.* 2010; Di Salvatore *et* *al.* 2008). Based on these previous findings, four Cu concentrations were added to the medium: 0 mg l^{-1} Cu (control), 5 mg l^{-1} Cu, 10 mg l^{-1} Cu and 20 mg l^{-1} Cu. After the copper (II) sulphate addition, the pH of the medium across treatments was 5.4 ± 0.1.

Due to the small population size and the IUCN status of the species, the three collected populations (ca. 1000 seeds per population) were pooled before the experiment. Seed lots of 20 seeds were weighed to the nearest 0.1 mg and received one of three disinfection treatments: bleach, fungicide or sterile water. The first was based on a widely used protocol of seed surface disinfection using 1.6% sodium hypochlorite (Sauer & Burroughs 1986). The second used a broad-spectrum anti-fungal agent (ROVRAL® SC, 2 % - BASF), and the third was the control (no disinfection treatment). Seeds were soaked for 5 minutes in either disinfection treatment followed by three rinses of 5 seconds in three different vessels of sterile water. Finally, seed lots were placed in Petri dishes having distinct copper concentrations. Ten replications were performed for each treatment combination (copper concentrations × disinfection) resulting in 120 dishes and 2400 seeds. The dishes were wrapped with Parafilm® and placed in an incubator at 25 °C with a 12 hour light/12 hour dark photoperiod. This corresponds to the natural conditions at the beginning of the growing season in Katanga. Photosynthetic photon flux density (PPFD) was set at 30 µmol m^{-2} s^{-1} using cool white fluorescent lamps (Philips TL 8W/33-640). This standard intensity used for germination tests is lower than the intensity in natural tropical areas. Germination was recorded and Petri dishes were randomized three times a week. Seeds were considered germinated when the radicle emerged.

Seed viability was measured with two cut tests. An initial cut test was performed on 65 seeds from the initial seed sample. A second cut test was performed on a subset of 10 non-germinated seeds randomly chosen from each treatment combination (4 copper concentrations × 3 disinfection treatments, for a total of 120 seeds) at the end of the experiment. The experiment duration was 30 days, based on mean germination time (MGT) observed by Godefroid *et al.* (2013) for this copper-endemic species.

A two-way ANCOVA was performed to compare the germination percentage between copper concentrations and disinfection treatments (as fixed factors) using seed mass (i.e. mass of lots of 20 seeds) as a covariate. To compare the percentage of seed viability, two separate one-way ANOVAs were performed among the copper concentrations or the disinfection treatments. Statistical analyses were performed using the statistical software R (R Development Core Team 2010).

An average final germination percentage of 15.2 ± 8.2% (Mean ± SD, %) was obtained. No significant effect of either fixed factor on final germination percentage was found (Fig. 1). The cumulative germination curves showed a similar germination rate over time among copper concentrations and disinfection treatments (Fig. 2).

The statistical analyses showed a significant effect of the covariate (seed mass) on final germination percentage ($F_{1,113} = 23.99$, $P < 0.001$), and a positive correlation between these two variables ($r = 0.422$, $P < 0.001$, N = 120). Heavier seed lots showed higher germination percentages. During the experiment, infection by fungi was observed on the surface of seeds, and more than 90% of seeds were affected regardless of the disinfection treatment or the copper concentration.

The cut test performed prior to germination showed that approximately half of the seeds (46.1%) contained a viable embryo. The second cut test carried out on non-germinated seeds showed that the viability of seeds was unaffected by disinfection treatments or copper concentrations (average viability of non-germinated seeds: 24.2 ± 10.8%).

A previous study on *D. marthozianum* showed that the percentage of germination was around 37.4 ± 35.0% (Mean ± SD, %) in non-copper-enriched substrate at 22 °C and under similar light intensity (Godefroid *et al.* 2013). However, this experiment used only viable seeds, whereas we did not sort viable seeds from seed lots. If we consider the seed lot viability of 46.1%, the germination percentage of fully developed seeds would be 32.9 ± 17.6%. In both cases, the germination percentage was similar and presented a high standard deviation that could be due to population variation, since Godefroid *et al.* (2013) also used three populations from different sites but found lower seed viability. Seed viability is particularly important to implement conservation and restoration programs in tropical areas (Bargali & Bargali 2016; Silveira *et al.* 2014)

Our results did not support the first hypothesis that the germination of *D. marthozianum* is limited by fungal attack in the

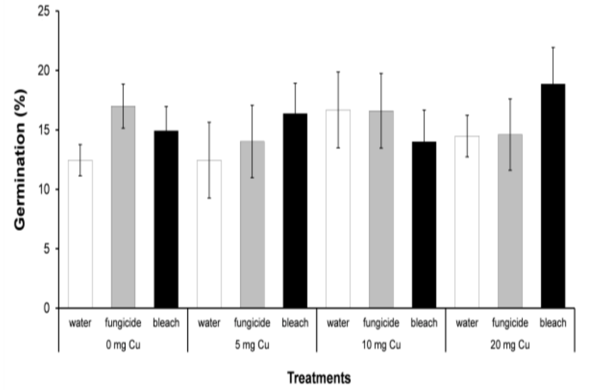

Fig. 1. Final percentage of germination (mean ± standard deviation, %) of the species *D. marthozianum* under different copper concentration and disinfection treatments.

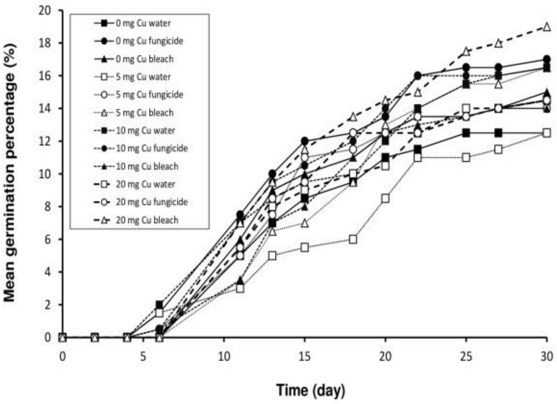

Fig. 2. Cumulative germination percentage of the species *D. marthozianum* under different copper concentration and disinfection treatments during 30 days.

absence of pathogen control by copper. The addition of copper in the medium did not hinder the growth of fungi, and fungi did not affect the germination percentage of *D. marthozianum*. In contrast, *Haumanisatrum katangense* has been identified as highly susceptible to pathogen attacks in non-copper-enriched soils (Chipeng *et al.* 2010). As copper is known as a mineral anti-fungal agent (Carlile *et al.* 2001), the presence of high levels of cupric ions in the soil may promote the selection of fungi possessing genetic determinants for copper resistance, mainly controlled by the cell wall (Cervantes & Gutiérrez-Corona 1994). The presence of pathogens in all media suggests that they were from the seed surface and microcavities of *D. marthozianum*. This form of fungi could be classified as seed-borne fungi that are dependent on seeds as a host for their dispersal and development (Carlile *et al.* 2001).

The hypothesis that germination of *D. marthozianum* requires high copper concentrations was not supported, since no significant differences in germination percentage were found among copper concentrations. However, either high copper requirements or low tolerance could occur at other developmental stages. Seedlings have been suspected to be more sensitive to metals than mature plants for some species (Street *et al.* 2007). Also, the exclusion of copper endemics from the dominant vegetation in soils that are not enriched in copper could be expected (i.e. edaphic endemism). This species response to copper in more advanced growth stages should be studied.

Seed mass was an important source of phenotypic variation among *D. marthozianum* that proportionally affected the germination independently of the treatments (i.e. copper concentrations or disinfection). This result suggests that maternal effects can play a significant role in germination dynamics and subsequent seedling traits, as already documented for other species (Monty *et al.* 2013; Roach & Wulf 1987). These results have direct implications for conservation strategies of this species regarding the selection of seeds for propagation in a nursery.

From a conservation perspective, our results indicate that the copper endemic *D. marthozianum* is not copper-dependent and that, although it is susceptible to fungal attack, this does not affect seed germination. Thus, disinfection of seeds does not seem to be a crucial step for the propagation of this species. In contrast, the selection of the largest seeds may ensure the highest germination success in a nursery. Apart from germination, future research should consider the growth and the survival of seedlings of *D. marthozianum*, which may be more sensitive to copper and to pathogens than seeds. A better understanding of the effect of copper concentration on the germination and development of copper endemic species is crucial for their *ex situ* conservation.

Acknowledgements

The authors would like to thank Julie Lebrun and Maxime Séleck, who collected the seeds used in the present study, and Cynthia Thomas for her help during the experimental manipulations. Sandrine Godefroid contributed to the implementation of the experiment and provided information about the species. This study was funded by the Fonds de la Recherche dans l'Industrie et dans l'Agriculture (FRIA) of the FNRS (Fonds National de la Recherche Scientifique, Belgium) and under FNRS Grant n°1.5061.12.

References

Antonovics, J., A. D. Bradshaw, & R. G., Turner. 1971. Heavy metal tolerance in plants. *Advances in Ecological Research* **7:** 1–85.

Baker, A. J. M., W. H. O. Ernst, A. Van der Ent, F. Malaisse & R. Ginocchio. 2010. Metallophytes: the unique biological resource, its ecology and conservational status in Europe, central Africa and Latin America. pp. 7–40. *In*: L. Batty & K. Hallberg (eds.). *Ecology of Industrial Pollution*. Cambridge University Press. Cambridge.

Bargali, K. & S. S. Bargali. 2016. Germination capacity of seeds of leguminous plants under water deficit conditions : implication for restoration of degraded lands in Kumaun Himalaya. *Tropical Ecology* **57:** 445–453.

Boyd, R. S. 2007. The defense hypothesis of elemental hyperaccumulation: status, challenges and new directions. *Plant and Soil* **293**: 153–176.

Bulajic, A., I. Djekic, N. Lakic & B. Krstic. 2009. The presence of *Alternaria* spp. on the seed of Apiaceae plants and their influence on seed emergence. *Archives of Biological Sciences* **61**: 871–881.

Carlile, M. J., S. C. Watkinson & G. W. Gooday. 2001. *The Fungi.* (Gulf Professionnal Publishing, Ed.). Academic Press. New York, N.Y., USA.

Cervantes, C. & F. Gutiérrez-Corona. 1994. Copper resistance mechanism in bacteria and fungi. *FEMS Microbiology Reviews* **14:** 121–138.

Chipeng, F. K., C. Hermans, G. Colinet, M. P. Faucon, M. Ngongo, P. Meerts & N. Verbruggen. 2010. Copper tolerance in the cuprophyte *Haumaniastrum katangense* (S. Moore) P.A. Duvign. & Plancke. *Plant and Soil* **328:** 235–244.

Di Salvatore, M., A. Carafa & G. Carratù. 2008. Assessment of heavy metals phytotoxicity using seed germination and root elongation tests: a comparison of two growth substrates. *Chemosphere* **73:** 1461–1464.

Duvigneaud, P. & S. Denaeyer-De Smet. 1963. Etudes sur la végétation du Katanga et de ses sols métallifères. Communication n°7 Cuivre et végétation au Katanga. *Bulletin de La Société Royale de Botanique de Belgique* **96**: 93–231.

Faucon, M. P., A. Meersseman, M. N. Shutcha, G. Mahy, M. N. Luhembwe, F. Malaisse, O. Poureet & P. Meerts. 2010. Copper endemism in the Congolese flora: a database of copper affinity and conservational value of cuprophytes. *Plant Ecology and Evolution* **143:** 5–18.

Faucon, M. P., F. Chipeng, N. Verbruggen, G. Mahy, G. Colinet, M. Shutcha & P. Meerts. 2012. Copper tolerance and accumulation in two cuprophytes of South Central Africa: *Crepidorhopalon perennis* and *C. tenuis* (Linderniaceae). *Environmental and Experimental Botany* **84**: 11–16.

Fones, H., C. R. Davis, A. Rico, F. Fang, J. A. C. Smith & G. M. Preston. 2010. Metal hyperaccumulation armors plants against disease. *PLoS Pathogens* **6:** 1–13.

Gankin, R. & J. Major. 1964. *Arctostaphylos myrtifolia*, its biology and relationship to the problem of endemism. *Ecology* **45:** 792–808.

Godefroid, S., A. Van de Vyver, W. Massengo Kalenga, G. Handjila Minengo, C. Rose, M. Ngongo Luhembwe, T. Vanderborght & G. Mahy. 2013. Germination capacity and seed storage behaviour of threatened metallophytes from the Katanga copper belt (DR Congo): implications for ex situ conservation. *Plant Ecology and Evolution* **146:** 183–192.

Kumari, S. & R. Ichhpujani. 2000. *Guidelines on standard operating procedures for Microbiology*. World Health Organization.

Monty, A., J. P. Bizoux, J. Escarré & G. Mahy. 2013. Rapid Plant Invasion in Distinct Climates Involves Different Sources of Phenotypic Variation. *PLoS ONE* **8:** 0055627.

Myers, N., R. Mittermeier, C. G. Mittermeier, G. da Fonseca & J. Kent. 2000. Biodiversity hotspots for conservation priorities. *Nature* **403:** 853–858.

Peng, H., Q. Wang-Müller, T. Witt, F. Malaisse & H. Küpper. 2012. Differences in copper accumulation and copper stress between eight populations of *Haumaniastrum katangense*. *Environmental and Experimental Botany* **79**: 58–65.

Pollard, A. J., K. D. Powell, F. A. Harper & J. A. C. Smith. 2002. The Genetic Basis of Metal Hyperaccumulation in Plants. *Critical Reviews in Plant Sciences* **21:** 539–566.

Primack, R. B. 2010. *Essentials of Conservation Biology*. Sinauer Associates, Incorporated.

R Development Core Team, 2010. *A Language and Environment for Statistical Computing*. Vienna (Austria).

Roach D. & R. D. Wulf. 1987. Maternal effects in plants. *Annual Review of Ecology and Systematics* **1987**: 209–235

Saad L., I. Parmentier, G. Colinet, F. Malaisse, M. P. Faucon, P. Meerts & G. Mahy. 2012. Investigating the Vegetation-Soil Relationships on the Copper-Cobalt Rock Outcrops of Katanga (D. R. Congo), an Essential Step in a Biodiversity Conservation Plan. *Restoration Ecology* **20**: 405–415.

Sauer D. & R. Burroughs. 1986. Disinfection of seed surfaces with sodium hypochlorite. *Phytopathology* **76**: 745–749.

Schemske D. W., B. C. Husband, M. H. Ruckelshaus, C. Goodwillie, I. M. Parker & J. G. Bishop. 1994. Evaluating Approaches to the Conservation of Rare and Endangered Plants. *Ecological Society of America* **75**: 584–606.

Silveira, F. A. O., D. Negreiros, B. D. Ranieri, C. A. Silva, L. M. Araujo & G. W. Fernandes. 2014. Effect of seed storage on germination, seedling growth and survival of Mimosa foliolosa (Fabaceae): Implications for seed banks and restoration ecology. *Tropical Ecology* **55**: 385–392.

Street R. A., M. G. Kulkarni, W. A. Stirk, C. Southway & J. Van Staden. 2007. Toxicity of metal elements on germination and seedling growth of widely used medicinal plants belonging to Hyacinthaceae. *Bulletin of Environmental Contamination and Toxicology* **79**: 371–376.

Tadros T. T. M. 1957. Evidence of the presence of an edapho-biotic factor in the problem of serpentine tolerance. *Ecology* **38**: 14–23.

UNEP. (2007). *Annual Report. United Nations Environment Programme.* Retrieved from http://www.unep.org/Documents.multilingual/Default.asp?DocumentID=67&ArticleID=5743&l=en.

Whiting S. N., R. D. Reeves & A. J. M. Baker. 2002. Conserving biodiversity: Mining, metallophytes and land reclamation. *Mining Environmental Management* **10**: 11–16.

(Received on 23.08.2014 and accepted after revisions, on 05.10.2015)